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Ploszaj et al., Amino acids (Austria), 2000, 19 (2), p483-96.

Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (10), p103-11.

Li et al. Am. journal of physiology , April 1999, 276 (4 Pt. 1), pC946-54.

Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704.

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular parhology (US), 1997, 64 (3), p147-55.

28 [Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p157-66..

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

## Neurotoxicity of Polyamines and Pharmacological Neuroprotection in Cultures of Rat Cerebellar Granule Cells

Mauro Sparapani,\* Rossella Dall'Olio,† Ottavio Gandolfi,† Elisabetta Ciani,\* and Antonio Contestabile\*

\*Department of Biology and †Department of Pharmacology, University of Bologna, 40126 Bologna, Italy

We have studied in a well-characterized *in vitro* neuronal system, cultures of cerebellar granule cells, the toxicity of polyamines endogenously present in the brain: spermine, spermidine, and putrescine. Twenty-four-hour exposure of mature (8 days *in vitro*) cultures to 1–500  $\mu M$  spermine resulted in a dose-dependent death of granule cells, with the half-maximal effect being reached below 50  $\mu M$  concentration. Putrescine was moderately toxic but only at 500  $\mu M$  concentration. Spermidine was tested at 50 and 100  $\mu M$  concentration and its toxicity was evaluated to be about 50% that of spermine. Neuronal death caused by spermine occurred, at least in part, by apoptosis. Spermine toxicity was completely prevented by competitive (CGP 39551) and noncompetitive (MK-801) antagonists of the NMDA receptor, but was unaffected by a non-NMDA antagonist (NBQX) or by antagonists of the polyamine site present on the NMDA receptor complex, such as ifenprodil. A partial protection from spermine toxicity was obtained through the simultaneous presence of free radical scavengers or through inhibition of the free radical-generating enzyme nitric oxide synthase, known to be partially effective against direct glutamate toxicity. The link between spermine toxicity and glutamate was further strengthened by the fact that, under culture conditions in which glutamate toxicity was ineffective or much reduced, spermine toxicity was absent or very much decreased. Exposure to spermine was accompanied by a progressive accumulation of glutamate in the medium of granule cell cultures. This was attributed to glutamate leaking out from dying or dead cells and was substantially prevented by the simultaneous presence of MK-801 or CGP 39551. The present results demonstrate that polyamines are toxic to granule cells in culture and that this toxicity is mediated through the NMDA receptor by interaction of exogenously added polyamines with endogenous glutamate released by neurons in the medium. The involvement of brain polyamines, in particular spermine and spermidine, in excitotoxic neuronal death is strongly supported by our present results. © 1997 Academic Press

### INTRODUCTION

Brain polyamines play an important role in brain maturation, particularly concerning the regulation of cell proliferation during neurogenesis (3, 27, 55, 56). Accordingly, the activity of the key enzyme ornithine decarboxylase (ODC) and the levels of its product, putrescine, are high at early stages of brain development and dramatically decrease during neonatal life (55, 56). However, relatively high concentrations of spermidine and spermine, the two polyamines following putrescine in the biosynthetic pathway, are maintained in the adult brain (52, 53, 56). An important aspect of the adult brain polyamine system is its response to neuropathological insults, when ODC is rapidly and transiently induced and putrescine levels increase soon thereafter (1, 4, 15, 16, 21, 31, 38, 41). Bringing together our knowledge on the developmental role of polyamines with that on injury response leads to somewhat intriguing and conflicting hypotheses. Polyamines may cooperate in reparative efforts of the nerve tissue (1, 15) or play a direct role in neurodegenerative processes (11, 31, 54, 58).

The recent discovery of a spermidine–spermine-positive modulatory site on the *N*-methyl-D-aspartate (NMDA) receptor subtype for glutamate (14, 43, 59) has generated great interest concerning its possible role in neuropathology related to an excessive stimulation of glutamate receptors, in particular, that of the NMDA subtype (39). In some paradigms of excitotoxic neuropathology, indeed, inhibition of ODC or blockade of the polyamine site on the NMDA receptor has been shown to be neuroprotective (5, 30, 36, 51, 57, 60). However, in cultures of cerebellar granule cells, inhibition of ODC was able to block the enzyme induction elicited by glutamate but unable to counteract glutamate excitotoxicity (33). Even more conflicting results stem from experiments in which the toxicity of polyamines was directly challenged and protection was sought through competitive or noncompetitive antagonists of the NMDA receptor. Dose-dependent neurotoxicity of spermine and spermidine in rat hippocampal slices was not counteracted by the NMDA channel blocker MK-801 or by the polyamine site antagonist ifenprodil (58). Intra-

striatal injections of spermine or spermidine in adult rats resulted in a dose-dependent degeneration of striatal tissue and this neurotoxic effect was not ameliorated by systemic administration of MK-801 (6, 58) or of an ifenprodil-related molecule, SL-82.0715 (eliprodil; 58). In a subsequent study, however, striatal NMDA toxicity in neonatal rats was found to be antagonized by intraparenchymal coadministration of both MK-801 or the polyamine inverse agonist 1,10-diaminodecane (36). An intriguing recent paper (37) reports protection from intrastriatal toxicity of spermine by intraparenchymal coadministration of either MK-801 or the non-NMDA receptor antagonist NBQX as well as by pretreatment with pentobarbital. In an *in vitro* model of cultured chick cortical neurons (23), spermine, but not spermidine, was toxic at very high doses and/or for extended exposure times and MK-801 was able to protect in at least one of the dose/time combinations tested. In cultures of hippocampal neurons, a short exposure to a very high concentration of spermine (1 mM for 10 min) resulted in delayed toxicity 24 h later, which was prevented by coexposure to MK-801 together with spermine but not by coexposure to ifenprodil (51). Noticeably, an equivalent exposure to spermine was found to be not toxic to cultures of cerebellar granule cells (33).

The presence of these conflicting results and the interest related to the characterization of polyamine toxicity prompted us to a reappraisal carried out on cultures of cerebellar granule cells, an *in vitro* system very well characterized with respect to glutamate toxicity and its pharmacology.

## MATERIALS AND METHODS

### Cell Cultures

Primary cultures of rat cerebellar granule cells were prepared from 8-day-old pups (Wistar) of both sexes as described previously (24, 45). Neurons dissociated from cerebella were plated at a density of  $2.4 \times 10^6$  cells/dish on 35-mm plastic dishes coated with poly-L-lysine (10  $\mu$ g/ml) and grown in basal modified Eagle's medium (BMME) containing 10% heat-inactivated fetal calf serum, 25 mM KCl (except when differently specified, see below), 2 mM glutamine and gentamicin (100  $\mu$ g/ml). Cultures were placed in a humidified incubator at 37°C under 95% air/5% CO<sub>2</sub> atmosphere until experiments were performed after 8 days *in vitro* (3 days in some experiments, see below). Glial proliferation was prevented by addition of 10  $\mu$ M cytosine arabinofuranoside as mitosis inhibitor, 16 h after plating.

### Cell Treatments and Viability Assay

Cultures were washed in BMME and exposed in fresh serum-free medium to the various polyamines

tested, alone or in the presence of other drugs for 24 h (4–24 h for the time-response curve and 16 h for shifting experiments, see below) under standard incubation conditions until the viability assay was performed. For shifting experiments, cultures were switched, after 8 days *in vitro* to serum-free BMME containing 5 mM K<sup>+</sup> and kept under these conditions for 16 h. In a further group of experiments, cultures were prepared from the beginning in a complete medium, containing 10 mM K<sup>+</sup>; these cultures were exposed to spermine (50  $\mu$ M) for 24 h in 10 mM K<sup>+</sup>-containing serum-free medium, after 8 days in culture.

Cytotoxicity and protection were quantitatively monitored by measuring lactate dehydrogenase (LDH) released by damaged cells, following a procedure similar to the one described previously (42). Four-hundred microliters, from 1 ml, of the medium was added to 2.4 ml of substrate solution ( $\beta$ -NADH 8 mg/100 ml; 14.4 mg/100 ml Na-pyruvate in 2 mM Tris-HCl, pH 7.4) and the absorbance at 340 nm was monitored every 20 s for 2 min (LDH in A). LDH activity was calculated from the slope of the absorbance curve. The cells adherent to the dish were lysed, without previous washing, with 1 ml of 0.1 M potassium phosphate buffer containing 0.5% Triton X-100, and 400  $\mu$ l of the lysate was used to measure LDH activity (LDH in B). Cell mortality was assessed by the percentage of released LDH, which is directly proportional to the number of dead cells, according to the formula

$$\% \text{ LDH release} = \frac{\text{LDH in A}}{\text{LDH in A} + \text{LDH in B}} \times 100.$$

In some cultures cell viability was visually monitored through a staining procedure (32) using fluorescein diacetate (FDA, 15  $\mu$ g/ml). Briefly cell cultures were washed in Locke's buffer (154 mM NaCl; 5.6 mM KCl; 3.6 mM NaHCO<sub>3</sub>; 2.3 mM CaCl<sub>2</sub>; 1.2 mM MgCl<sub>2</sub>; 5.6 mM glucose; 5 mM Hepes; pH 7.4) and then stained for 3 min. After staining, the cultures were washed again in Locke's buffer and observed with a fluorescence microscope, equipped with a standard filter system. Viable cells appear bright green when observed under these conditions. Other cultures were fixed in 4% paraformaldehyde and stained with the nuclear stain Hoechst 33258. Part of the fixed cultures were used to reveal apoptotic cell death by specific *in situ* detection of fragmented DNA using a modification of the terminal transferase-mediated dUTP nick end labeling (TUNEL) method (25). After washing with PBS, cells were permeabilized for 2 min at 4°C with 0.1% Triton X-100 in 0.1% sodium citrate, washed again in PBS, and incubated for 60 min at 37°C in a humidified chamber in the dark in a mixture containing the enzyme and fluorescein-labeled nucleotide (*in situ* cell death detection kit, fluorescein, Boehringer). After being washed with PBS, dishes were

observed and photographed with the fluorescence microscope.

#### Determination of Glutamate Content and Release of D-[<sup>3</sup>H]Aspartate in the Medium

After 4, 8, 16, or 24 h of exposure to spermine alone or together with other drugs, aliquots of the medium were collected and centrifuged for 15 min at 11,000 rpm in an Eppendorf centrifuge. The samples were then analyzed by HPLC (28) for the determination of amino acid content, using a precolumn derivatization procedure with *O*-phthaldialdehyde and a reverse-phase column, elution being performed with a linear gradient of 19 to 80% methanol in acetate buffer (pH 6.8). To investigate whether a predamage release of glutamate could be directly stimulated by spermine, cultures at 8 days *in vitro* were incubated for 2 h with 1  $\mu$ M D-[<sup>3</sup>H]aspartate (a nonmetabolized analog of glutamate which is taken up and released by glutamatergic neurons). The cultures were then washed three times with medium and incubated again in serum-free medium in the presence or absence of 50  $\mu$ M spermine. Samples (50  $\mu$ l) of the medium were collected at various intervals between 1 and 4 h and counted for radioactivity.

#### Statistical Analysis

Statistical analysis of the results was carried on by using parametric methods, after having assessed the normal distribution of the data to be compared. Significant differences between the various experimental conditions were evaluated through the analysis of variance (one-way ANOVA), followed by post hoc comparisons (Bonferroni's test), or, when appropriate, through the Student *t* test.

#### Chemicals

Culture media and fetal calf serum were from Gibco. Spermine tetrahydrochloride, spermidine tetrahydrochloride, putrescine dihydrochloride, poly-L-lysine, gentamicin, cytosine arabino-furanoside, ifenprodil, *N*-nitro-L-arginine (NARG), and butylated hydroxy anisole (BHA) were purchased from Sigma Chemical Co. (St. Louis, MO); (+)-MK-801 hydrogen maleate, a potent blocker of the NMDA receptor-associated ionic channel, and *N*-methyl-D-aspartate were obtained from Research Biochemical International. CGP 39551, an effective competitive NMDA antagonist (49), was a generous gift from Ciba-Geigy (Switzerland). NBQX, a selective AMPA/kainate antagonist, was kindly supplied by Novo Nordisk (Denmark) and SL 82.0715 (eliprodil), an antagonist of the polyamine site on the NMDA receptor, was supplied by Synthelabo (France). Drugs were dissolved in water (containing 1  $M$  malic acid in the case of SL 82.0715) as concentrated solutions and diluted in the culture medium so that not

more than 1% of solution or vehicle was added. Polyamines were directly dissolved in the culture medium; the pH of the solutions was adjusted and the medium equilibrated in the incubator before use.

## RESULTS

When, after 8 days *in vitro*, cultures of cerebellar granule cells were challenged with polyamines for 24 h, a substantial toxicity was displayed by spermine on the basis of LDH released from dead cells. An approximately half-maximal toxicity was attained below 50  $\mu$ M spermine (the concentration causing 50% lethality, LC<sub>50</sub>, was 34.9  $\mu$ M), while the effect of 100  $\mu$ M concentration was almost maximal (Fig. 1). On the contrary, putrescine showed only moderate toxicity even at very high (500  $\mu$ M) concentration (Fig. 1). The time course of 50  $\mu$ M spermine toxicity was followed at various intervals up to 24 h and showed an almost linear increase from 4 to 16 h with a small, but not significant further increase between 16 and 24 h (Fig. 1).

Figures 2A and 2B demonstrate that a 24-h exposure to 50  $\mu$ M spermine resulted in a large depletion of granule cells from the cultures compared to controls. When cultures were exposed to spermine together with the noncompetitive antagonist of the NMDA receptor MK-801 (5  $\mu$ M) there was protection of granule cells from death (Fig. 2C). An almost equivalent result was obtained using the competitive NMDA antagonist CGP 39551 (50  $\mu$ M) (Fig. 2D). Spermidine appeared somewhat less potent than spermine: a large depletion of granule cells after a 24-h exposure was only obtained using 100  $\mu$ M spermidine, with 50  $\mu$ M spermidine being considerably less effective (not shown). Also the toxicity of 100  $\mu$ M spermidine was effectively counteracted by NMDA receptor antagonists (not shown). Hoechst stain showed that in spermine-treated cultures some cell nuclei were abnormally condensed compared to controls (Figs. 3A and 3B). Specific *in situ* labeling of DNA fragmentation by the TUNEL method revealed that a sizable proportion of granule neurons was killed by spermine through apoptosis (Figs. 3C and 3D).

The protective effect of NMDA receptor antagonists was quantitatively confirmed by using LDH assay as a marker for spermine toxicity. As shown by Fig. 4, both 5  $\mu$ M MK-801 and 50  $\mu$ M CGP 39551, in the presence of 50  $\mu$ M spermine, gave values of percentage of LDH released from dead cells not significantly different from controls, while an antagonist of non-NMDA receptor subtypes, NBQX (50  $\mu$ M), did not change the spermine toxic effect. Spermine toxicity was not significantly counteracted by antagonists of the polyamine site present on the NMDA receptor (Fig. 4). Ifenprodil was ineffective at 10 and 50  $\mu$ M concentration, higher doses being toxic per se (not shown). A molecule of similar

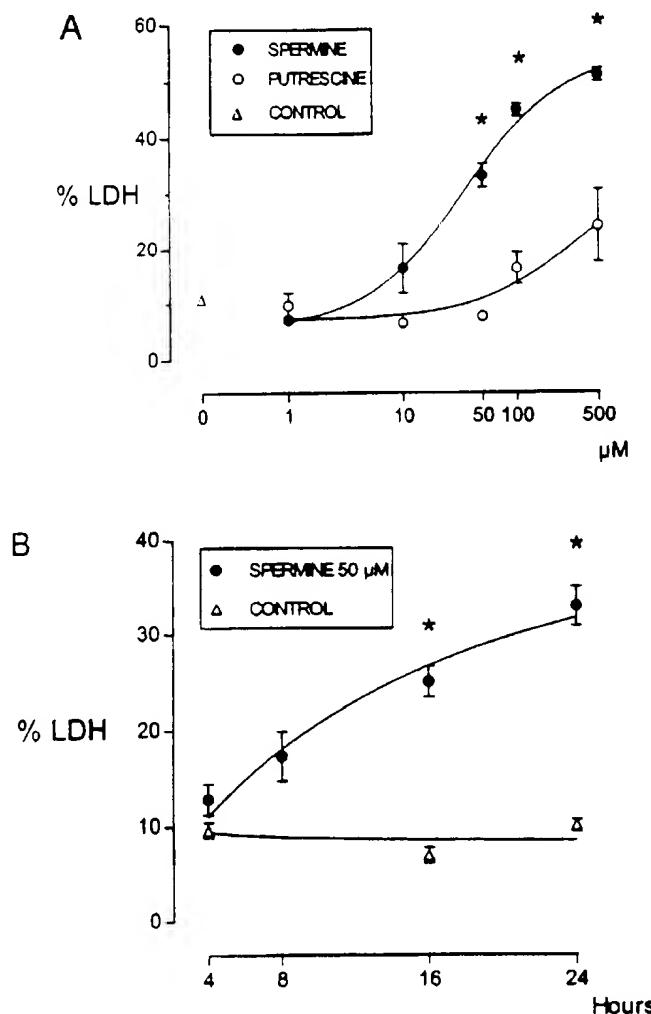


FIG. 1. (A) Effect of spermine and putrescine on cerebellar granule cell mortality, evaluated through percentage of LDH release after incubation with increasing doses of polyamines (1–500  $\mu$ M). After 8 days *in vitro*, cultures of cerebellar granule cells were exposed for 24 h to fresh serum-free medium containing the different concentrations of polyamines. Results are the mean  $\pm$  SEM of 7–53 dishes from at least three different cultures. \* $P$  < 0.01 vs control condition: Bonferroni's test after ANOVA. (B) Effect of various times of exposure to 50  $\mu$ M spermine on cerebellar granule cell mortality. After 8 days *in vitro*, cultures of cerebellar granule cells were exposed for 4–24 h to fresh serum-free medium containing 50  $\mu$ M spermine. Results are the mean  $\pm$  SEM of 6–53 dishes from at least three different cultures. \* $P$  < 0.01 vs control condition: Bonferroni's test after ANOVA.

structure, SL 82.0715 (eliprodil), was also ineffective against spermine toxicity at 50 and 100  $\mu$ M concentration, with the latter dose being toxic *per se* (Fig. 4).

Because it has been shown that in granule cell cultures the NMDA-mediated excitotoxic death can be partially counteracted through the use of free radical scavengers or by inhibiting free radical-generating enzymes (12) we tested whether this could also apply to spermine-related toxicity. As shown in Fig. 5 the toxic effect of 50  $\mu$ M spermine was slightly antagonized by the inhibition of a free radical-generating enzyme,

nitric oxide synthase (NOS) with NARG (100  $\mu$ M) but, more efficiently, by the use of two chain-breaking antioxidants: vitamin E (100  $\mu$ M) and in particular BHA (100  $\mu$ M).

As the above findings suggested a link between spermine toxicity and glutamate, a series of experiments was undertaken in order to substantiate this using immature cell cultures (3 days *in vitro*) in which excitotoxic sensitivity to glutamate had not yet developed or was strongly reduced by the culture conditions employed (45). When such immature cultures were exposed to glutamate (50  $\mu$ M) or spermine (50  $\mu$ M) for 24 h, no significant toxicity was apparent (%LDH: control, 10.4  $\pm$  1.4; glutamate, 17.8  $\pm$  2.6; spermine, 13.9  $\pm$  2.0;  $n$  = 6, differences not significant by ANOVA). Furthermore, in cultures grown for 8 days in a medium containing 10 mM K<sup>+</sup>, a condition which substantially prevents the development of sensitivity to glutamate toxicity (45), exposure to 50  $\mu$ M spermine was only slightly toxic (%LDH: control, 15.8  $\pm$  0.8; spermine, 19.3  $\pm$  0.6,  $n$  = 6). This was a mere 22% increase in percentage of LDH released versus 300% in the 25 mM K<sup>+</sup> condition (compare with Fig. 1).

To further exploit the relationship between K<sup>+</sup>-maintained depolarization and spermine toxicity, cultures grown for 8 days in 25 mM K<sup>+</sup>-containing medium were shifted to 5 mM K<sup>+</sup>-containing medium and percentage of LDH released was measured 16 h later in order to minimize the apoptotic granule cell death due to shifting from high to low K<sup>+</sup> (13, 18). Under these conditions, no additional toxicity due to spermine exposure was measured over the granule cell mortality related to the change from trophic (depolarizing) to nontrophic (not depolarizing) conditions (Fig. 6). Instead, the presence of spermine appeared to somewhat protect granule cells from death caused by the shifting.

To additionally evaluate the role of excitatory amino acids in spermine toxicity, we measured the levels of glutamate in the serum-free culture medium under different experimental conditions and over a time span ranging from 4 to 24 h. Since no glutamate is included in the basic composition of the medium used, the accumulation measured after different times from medium change obviously reflects the release from cells related either to physiological mechanisms and/or to bulk release from damaged or dead cells. The results indicated that exposure to spermine resulted in a progressive increase in glutamate concentration in the culture medium and that this process was substantially prevented by coexposure to NMDA receptor antagonists (Fig. 7). Cultures at 8 days *in vitro* incubated for 2 h with 1  $\mu$ M D-[<sup>3</sup>H]aspartate did not demonstrate significant differences in its release during the following 4 h, when exposed or not exposed to 50  $\mu$ M spermine (not shown).

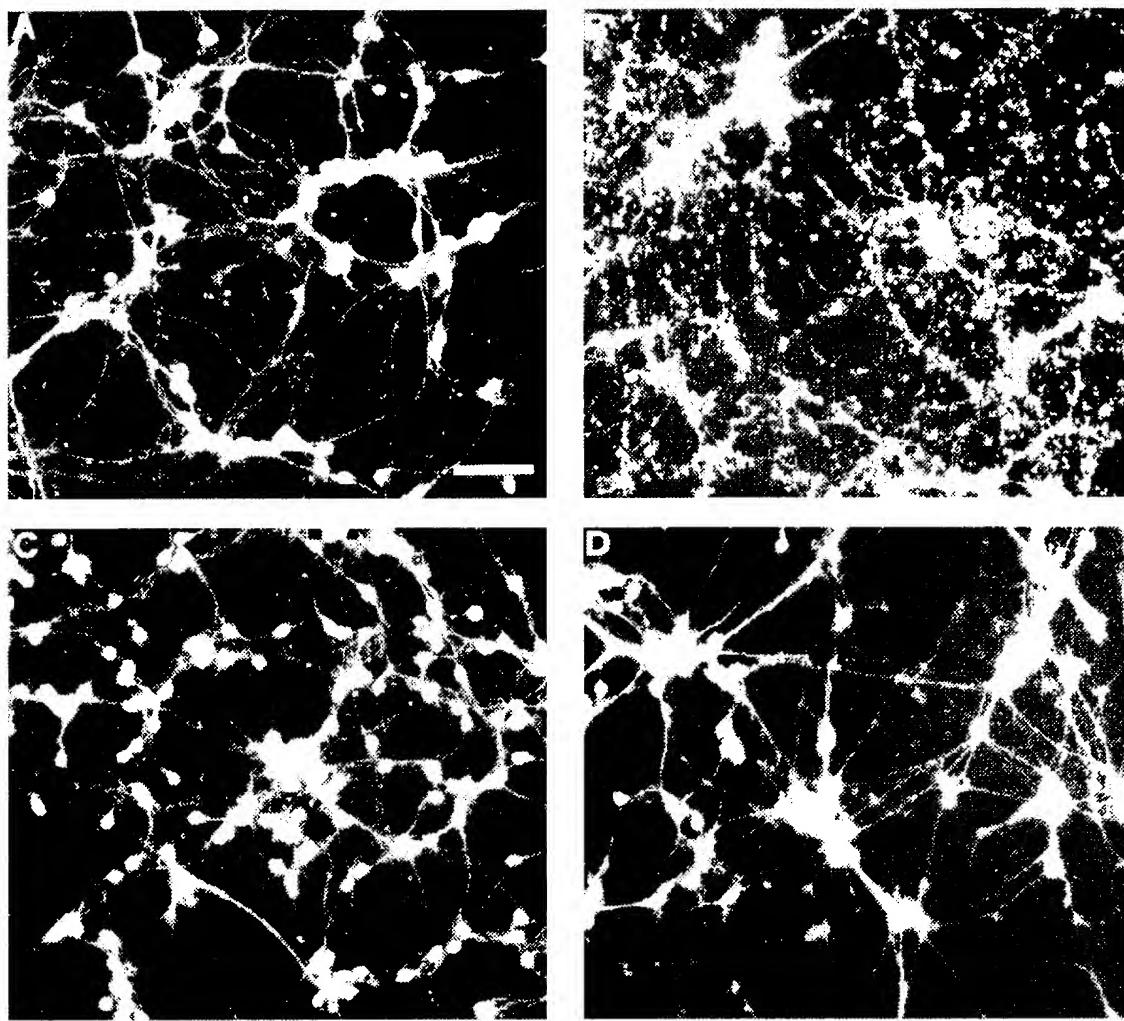


FIG. 2. Effect of spermine on granule cell viability and protection by NMDA-receptor antagonists. After 8 days *in vitro*, drugs were added in a small volume to the original serum-containing medium and dishes were stained with FDA and photographed 24 h later. (A) Control; (B) 0  $\mu$ M spermine; (C) 50  $\mu$ M spermine plus 5  $\mu$ M MK-801; (D) 50  $\mu$ M spermine plus 50  $\mu$ M CGP 39551. Bar, 30  $\mu$ m.

## DISCUSSION

In the present report we have shown that in a well-characterized *in vitro* culture system of neuronal cells, spermine, and to a lesser extent spermidine, is neurotoxic. We have also demonstrated that this neurotoxicity involves glutamate and can be blocked by antagonists to the NMDA receptor subtype. Furthermore, apoptosis contributed to neuronal death under these conditions.

From the present study, granule cells in culture appeared noticeably sensitive to spermine toxicity. In the only previous study in which nerve cells in culture were chronically exposed to spermine (23), chick forebrain neurons were much less sensitive, with a significant neurotoxic effect being attained only after a 24-h exposure to 500  $\mu$ M spermine. On the other hand, previous data showing spermine toxicity in hippocampal neurons (51) or no toxicity in cerebellar granule cells (33) are not directly comparable with the present

results since they were obtained through short (10 or 15 min) exposure to very high (up to 1 mM) concentrations of spermine in the presence (51) or in the absence (33) of physiological concentrations of  $Mg^{2+}$ .

Our present results demonstrate a link between polyamine neurotoxicity and glutamate acting at the NMDA receptor, since competitive and noncompetitive antagonists of the NMDA receptor, CGP 39551 and MK-801, were able to counteract spermine and spermidine toxicity. Additional indirect evidence linking spermine toxicity to glutamate comes from our results demonstrating that spermine toxicity is not effective or is very much reduced when granule cells have not yet acquired a full excitotoxic sensitivity to glutamate. Finally, a partial protection was obtained by adding free radical scavengers or by inhibiting NOS, similarly to that obtained against excitotoxicity directly mediated by glutamate (12).

Previous results (17, 24), as well as our present data,

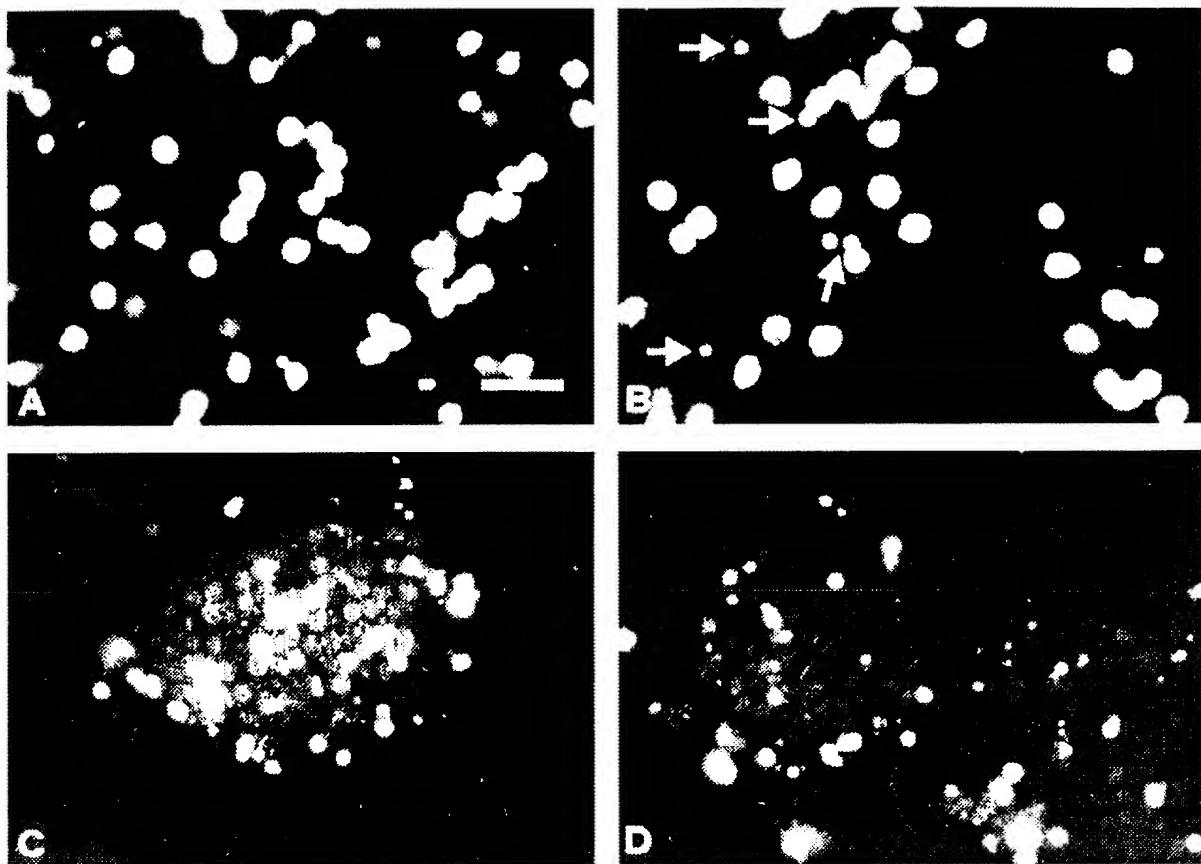
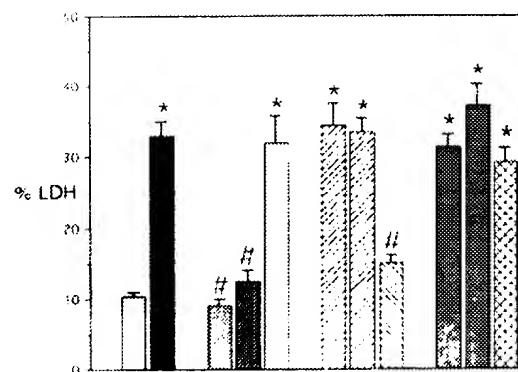


FIG. 3. Hoechst stain of a control culture after 8 days *in vitro* (A) and after a 16-h exposure to 50  $\mu\text{M}$  spermine (B). Arrows point to condensed nuclei. *In situ* labeling of apoptotic cells through TUNEL method after a 16-h exposure to 50  $\mu\text{M}$  spermine (C, D). Bar: A and B, 15  $\mu\text{m}$ ; C and D, 20  $\mu\text{m}$ .

show that glutamate is slowly accumulated in the medium of cultured granule cells until a steady concentration is reached, depending on the balance between release and uptake of the transmitter. The increase in glutamate accumulation in the medium of cultures exposed to spermine could have been attributed to a direct action favoring release and/or inhibiting uptake. The first possibility is excluded by our data which show no increase of the release of exogenously accumulated D-[<sup>3</sup>H]aspartate during the first hours of culture exposure to spermine. Concerning the second possibility, there are to our knowledge no data in the literature supporting it. It seems, therefore, reasonable to conclude that the toxic effect of spermine relies on its interaction with glutamate spontaneously released by cells in the medium. Previous research (33) demonstrated that exposure of granule cells in cultures to nontoxic doses of glutamate were made neurotoxic by the simultaneous addition of spermine to the medium. In our system, once the process of neural damage is triggered by the simultaneous presence of endogenously released glutamate and exogenously added spermine, it may go on in a cooperative way, with the increasing glutamate accumulation being due to glutamate leaking out from dying or dead cells.

The scenario derived from this *in vitro* system may be relevant to our understanding of similar processes *in vivo* and may explain why, under certain circumstances, polyamines contribute to neural damage (39). A toxic *in vivo* action of spermidine and spermine after icv administration was first demonstrated by Anderson (2). In replicating his experiments, we have additionally noticed that toxicity of icv-injected spermidine was significantly decreased by simultaneous administration of MK-801 (unpublished observations). It may be questioned whether the doses of polyamines found to be toxic *in vitro* approach concentrations occurring *in vivo* under pathological circumstances. The sum of the concentrations of spermidine and spermine in the brain is in the high micromolar range (52, 55). While this relatively high concentration largely reflects intracellular stores of polyamines, there is much evidence suggesting that a relevant release does occur under excitatory and excitotoxic conditions (10, 22, 26, 40). It is, therefore, conceivable that extracellular levels of toxic polyamines may reach concentrations that, when interacting with the NMDA receptor, contribute to the development of neuropathology.

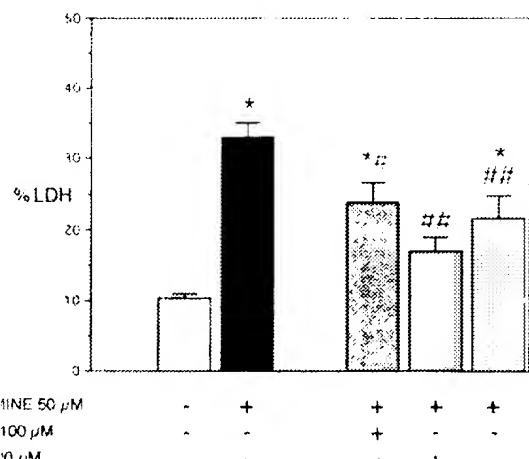
Having established the link between spermine neurotoxicity and glutamate acting through the NMDA recep-



	-	+	+	+	+	+	+	+	-
SPERMINE 50 $\mu$ M	-	+	+	+	+	+	+	+	-
MK 801 5 $\mu$ M	-	-	+	-	-	-	-	-	-
CGP 39551 50 $\mu$ M	-	-	-	+	-	-	-	-	-
NBQX 50 $\mu$ M	-	-	-	-	+	-	-	-	-
Ifenprodil 10 $\mu$ M	-	-	-	-	-	+	-	-	-
Ifenprodil 50 $\mu$ M	-	-	-	-	-	-	+	+	-
SL 82.0715 50 $\mu$ M	-	-	-	-	-	-	-	+	-
SL 82.0715 100 $\mu$ M	-	-	-	-	-	-	-	-	+

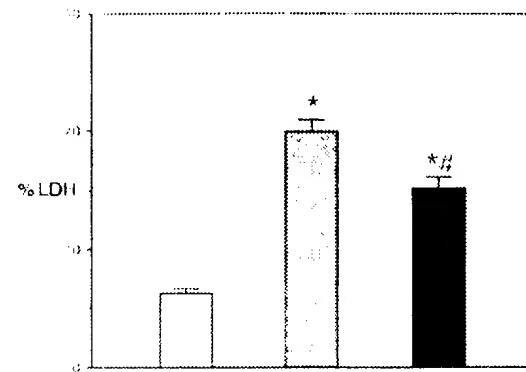
FIG. 4. Effect on spermine toxicity of two different NMDA-receptor antagonists (MK-801, CGP 39551), one non-NMDA receptor antagonist (NBQX) and two polyamine site antagonists (ifenprodil, SL 82.0715). After 8 days *in vitro*, cultures of cerebellar granule cells were exposed for 24 h to fresh serum-free medium containing 50  $\mu$ M spermine alone or together with the different drugs tested. Results are the mean  $\pm$  SEM of 10–53 dishes from at least four different cultures. \* $P$  < 0.01 vs control condition; # $P$  < 0.01 vs 50  $\mu$ M spermine; Bonferroni's test after ANOVA.

tor, the likely mechanism of interaction between spermine and the receptor itself responsible for neurotoxicity remains to be discussed. The most obvious reason, i.e., the positive modulation exerted through the ifenprodil-sensitive spermine–spermidine site present in the



	-	+	+	+	+
SPERMINE 50 $\mu$ M	-	+	+	+	+
NARG 100 $\mu$ M	-	-	+	-	-
BHA 100 $\mu$ M	-	-	-	+	-
Vit E 100 $\mu$ M	-	-	-	-	+

FIG. 5. Effect on spermine toxicity of a NOS inhibitor (NARG) and two chain-breaking antioxidants (vitamin E and BHA). After 8 days *in vitro*, cultures of cerebellar granule cells were exposed for 24 h to fresh serum-free medium containing 50  $\mu$ M spermine alone or together with the different drugs tested. Results are the mean  $\pm$  SEM of 14–53 dishes from at least four different cultures. \* $P$  < 0.01 vs control; # $P$  < 0.05 vs spermine alone; ## $P$  < 0.01 vs spermine alone; Bonferroni's test after ANOVA.



	+	-	-
Shifted to K 25 mM	+	-	-
Shifted to K 5 mM	-	+	+

FIG. 6. Effect of spermine on cultures shifted from trophic to nontrophic conditions. After 8 days of culture in 25 mM K<sup>+</sup>-containing medium, cells were shifted for 16 h to serum-free fresh medium with either 25 mM K<sup>+</sup> or 5 mM K<sup>+</sup> containing or not 50  $\mu$ M spermine. Results are the mean  $\pm$  SEM of 12–13 dishes from three different cultures. \* $P$  < 0.01 vs control; # $P$  < 0.05 vs shifted condition without spermine; Bonferroni's test after ANOVA.

NMDA receptor (5, 9, 47, 50), can be excluded since potent antagonists of this site, such as ifenprodil and SL 82.0715, were unable to counteract spermine toxicity in granule neurons in culture. This negative result correlates well with the fact that the NR2B subunit of the NMDA receptor, which binds spermine and related molecules (61), is downregulated in mature granule cells in culture (46). The toxicity of high doses of antagonists of the polyamine site on the NMDA receptor may be a side effect peculiar to cerebellar granule neurons. It should, however, be taken into consider-

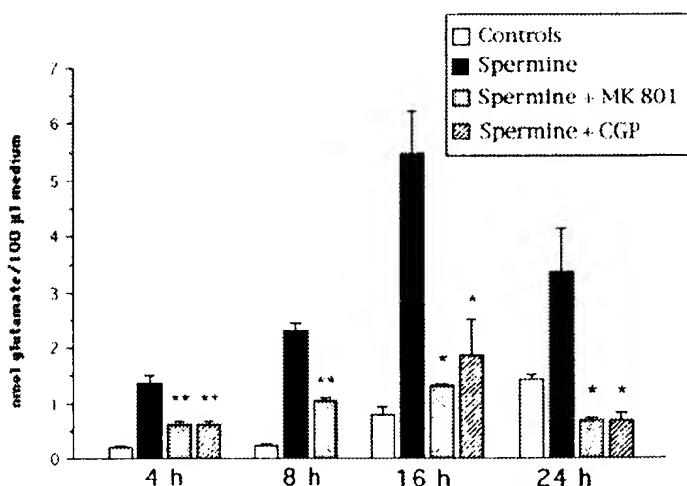


FIG. 7. Levels of glutamate in the culture medium at different times after exposure to fresh serum-free medium containing 50  $\mu$ M spermine alone or together with 5  $\mu$ M MK-801 or 50  $\mu$ M CGP 39551 (not determined after 8 h). Bars are the mean  $\pm$  SEM of three to six experiments. \* $P$  < 0.05, \*\* $P$  < 0.01 vs 50  $\mu$ M spermine; Student's *t* test.

ation when proposing the potential use of these and related drugs in preventing neurodegeneration (9).

Polyamines may interact with the NMDA receptor through different sites (7, 35, 44, 47, 48). A finding that may be relevant to understanding the present results is the reported competition between polyamines and  $Mg^{2+}$  for binding site(s) on the NMDA receptor (7, 48). While granule cells in culture are kept under conditions of partial depolarization,  $Mg^{2+}$  block of the NMDA receptor is likely to be effective to a significant extent (otherwise granule cells would be killed by glutamate produced and released in the medium by themselves). In addition to the above-mentioned interactions of polyamines with the NMDA receptor, negative interactions leading to functional inhibition, or to activation/inhibition depending on concentration, have been reported (35, 44, 47). Further interactions of polyamines with non-NMDA receptor channels as well as with some subtypes of potassium channels have recently been reported (8, 19, 20, 29, 34); these may play an additional role in polyamine toxicity. When the full extent of the involvement of polyamines in brain function is more completely understood, the role of these intriguing molecules in brain pathology will be better appreciated.

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#### REFERENCES

1. Agnati, L. E., K. Fuxe, P. Davalli, A. Corti, and M. Zoli. 1985. Striatal ornithine decarboxylase activity following neurotoxic and mechanical lesions of the mesostriatal dopamine system of the male rat. *Acta Physiol. Scand.* **125**: 173-175.
2. Anderson, D. J., J. Crossland, and G. G. Shaw. 1975. The actions of spermidine and spermine on the central nervous system. *Neuropharmacology* **14**: 571-577.
3. Bachrach, U. 1973. *Function of Naturally Occurring Polyamines*. Academic Press, New York.
4. Baudry, M., G. Lynch, and C. Galli. 1986. Induction of ornithine decarboxylase as a possible mediator of seizure-induced changes in genomic expression in rat hippocampus. *J. Neurosci.* **6**: 3430-3435.
5. Beart, P. M., A. Schousboe, and A. Frandsen. 1995. Blockade by polyamine NMDA antagonists related to ifenprodil of NMDA-induced synthesis of cyclic GMP, increases in calcium and cytotoxicity in cultured neurones. *Br. J. Pharmacol.* **114**: 1359-1364.
6. Bourdiol, F., D. Fage, A. Serrano, C. Carter, J. Benavides, and B. Scatton. 1992. Neurotoxic effects of the intrastratal injection of spermine and spermidine: Lack of involvement of NMDA receptors. *Brain Res.* **596**: 183-188.
7. Bowe, M. A., and J. W. Nadler. 1995. Polyamines antagonize N-methyl-D-aspartate-evoked depolarizations, but reduce  $Mg^{2+}$  block. *Eur. J. Pharmacol.* **278**: 55-65.
8. Bowie, D., and M. L. Mayer. 1995. Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* **15**: 453-462.
9. Carter, C., J. Benavides, C. Dana, H. Schoemaker, G. Perrault, D. Sanger, and B. Scatton. 1991. Noncompetitive NMDA receptor antagonists acting on the polyamine site. In *Excitatory Amino Acid Antagonists* (B. S. Meldrum, Ed.), pp. 130-163. Blackwell, Oxford.
10. Carter, C., H. Poignet, S. Carboni, D. Fage, C. Voltz, and B. Scatton. 1995. Release of spermidine from the rat cortex following permanent middle cerebral artery occlusion. *Fundam. Clin. Pharmacol.* **9**: 129-140.
11. Ciani, E., and A. Contestabile. 1993. Ornithine decarboxylase is differentially induced by kainic acid during brain development in the rat. *Dev. Brain Res.* **71**: 258-260.
12. Ciani, E., L. Groneng, M. Voltattorni, R. Veslemoy, A. Contestabile, and R. E. Paulsen. 1996. Inhibition of free radical production or free radical scavenging protect from excitotoxic cell death mediated by glutamate in cultures of cerebellar granule neurons. *Brain Res.* **768**: 1-6.
13. Ciani, E., S. Rizzi, R. E. Paulsen, and A. Contestabile. 1997. Chronic pre-explant blockade of the NMDA receptor affects survival of cerebellar granule cells explanted in vitro. *Dev. Brain Res.* **99**: 112-117.
14. Dana, C., J. Benavides, H. Shoemaker, and B. Scatton. 1991. Pharmacological characterization and autoradiographic distribution of polyamine-sensitive  $^3H$  ifenprodil binding sites in the rat brain. *Neurosci. Lett.* **125**: 45-48.
15. Desiderio, M. A., I. Zini, P. Davalli, M. Zoli, A. Corti, K. Fuxe, and L. E. Agnati. 1988. Polyamines, ornithine decarboxylase and diamine oxidase in the substantia nigra and striatum of the male rat after hemitransection. *J. Neurochem.* **51**: 25-31.
16. De Vera, N., E. Artigas, J. Serratosa, and E. Martinez. 1991. Changes in polyamine levels in rat brain after systemic administration of kainic acid: Relationship to convulsant activity and brain damage. *J. Neurochem.* **57**: 1-8.
17. Didier, M., N. Bursztajn, E. Adamec, L. Passani, R. A. Nixon, J. T. Coyle, J. Y. Wei, and S. A. Berman. 1996. DNA strand breaks induced by sustained glutamate excitotoxicity in primary neuronal cultures. *J. Neurosci.* **16**: 2238-2250.
18. D'Mello, S. R., C. Galli, T. Ciotti, and P. Calissano. 1993. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc. Natl. Acad. Sci. USA* **90**: 10989-10993.
19. Donevan, S. D., and M. A. Rogawski. 1995. Intracellular polyamines mediated inward rectification of  $Ca^{2+}$ -permeable  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Proc. Natl. Acad. Sci. USA* **92**: 9298-9302.
20. Fakler, B., U. Brändle, E. Glowatzki, S. Weidemann, H. P. Zenner, and J. P. Ruppertsberg. 1995. Strong voltage-dependent inward rectification of inward rectifier  $K^+$  channels is caused by intracellular spermine. *Cell* **80**: 149-154.
21. Facchinetto, F., M. Virgili, P. Migani, O. Barnabei, and A. Contestabile. 1992. Induction of brain ornithine decarboxylase after systemic or intrastratal administration of kainic acid. *Neurosci. Lett.* **140**: 59-62.
22. Fage, D., C. Voltz, B. Scatton, and C. Carter. 1992. Selective release of spermine and spermidine from the rat striatum by

*N*-methyl-D-aspartate receptor activation in vivo. *J. Neurochem.* **58**: 2170-2175.

23. Fahey, J. M., G. A. Pritchard, and L. G. Miller. 1993. Polyamine neurotoxicity is antagonized by dizolcipine in cultured chick cortical neurons. *Neurosci. Lett.* **161**: 109-112.
24. Gallo, V., M. T. Ciotti, A. Coletti, F. Aloisi, and G. Levi. 1982. Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. USA* **79**: 7919-7923.
25. Gavrieli, Y., Y. Sherman, and A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**: 493-501.
26. Harman, R. J., and G. G. Shaw. 1981. The spontaneous and evoked release of spermine from rat brain in vivo. *Br. J. Pharmacol.* **73**: 165-174.
27. Heby, O. 1981. Role of the polyamines in the control of cell proliferation and differentiation. *Differentiation* **19**: 1-20.
28. Hill, D. W., F. H. Walters, T. D. Wilson, and D. S. James. 1979. High performance liquid chromatographic determination of amino acids in the picomole range. *Anal. Chem.* **51**: 1338-1341.
29. Isa, T., M. Lino, S. Itazawa, and S. Ozawa. 1995. Spermine mediates inward rectification of  $\text{Ca}^{++}$ -permeable AMPA receptor channels. *Neuroreport* **6**: 2045-2048.
30. Fish, S. J., J. M. Wilson, and P. J. Fletcher. 1991. The polyamine synthesis inhibitor alpha-difluoromethylornithine is neuroprotective against *N*-methyl-D-aspartate-induced brain damage in vivo. *Eur. J. Pharmacol.* **209**: 101-103.
31. Koenig, H., A. D. Goldstone, and Y. L. Chung. 1989. Blood-brain barrier breakdown in cold-injured brain is linked to a biphasic stimulation of ornithine decarboxylase activity and polyamine synthesis: Both are coordinately inhibited by verapamil, dexamethasone and aspirin. *J. Neurochem.* **52**: 101-109.
32. Jones, K. H., and J. A. Senft. 1985. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J. Histochem. Cytochem.* **33**: 77-79.
33. Lombardi, G., A. M. Szekely, L. A. Bristol, A. Guidotti, and H. Manev. 1993. Induction of ornithine decarboxylase by *N*-methyl-D-aspartate receptor activation is unrelated to potentiation of glutamate excitotoxicity by polyamines in cerebellar granule neurons. *J. Neurochem.* **60**: 1317-1324.
34. Lopatin, N. A., E. N. Makhina, and C. G. Nichols. 1994. Potassium channels block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* **372**: 366-339.
35. Marvizon, J. C., and M. Baudry. 1994.  $^3\text{H}$ -Dizolcipine association kinetics distinguish stimulatory and inhibitory polyamine sites of *N*-methyl-D-aspartate receptors. *J. Neurochem.* **63**: 963-971.
36. Munir, M., M. Subramaniam, and P. McGonigle. 1993. Polyamine modulate the neurotoxic effects of NMDA in vivo. *Brain Res.* **616**: 163-170.
37. Otsuki, M., M. Davidson, S. Goodenough, P. A. Wilce, C. Tase, and I. Matsumoto. 1995. In vivo pharmacological study of spermine-induced neurotoxicity. *Neurosci. Lett.* **196**: 81-84.
38. Paschen, W., G. Rohn, C. O. Meese, B. Djuricic, and R. Schmidt-Kastner. 1988. Polyamine metabolism in reversible cerebral ischemia: Effect of alpha-fluoromethylornithine. *Brain Res.* **453**: 9-16.
39. Paschen, W. 1992. Polyamine metabolism in different pathological states of the brain. *Mol. Chem. Neuropathol.* **16**: 506-520.
40. Paschen, W., R. Widmann, and C. Weber. 1992. Changes in regional polyamine profile in rat brain after transient cerebral ischemia (single versus repetitive ischemia): Evidence for release of polyamines from injured neurons. *Neurosci. Lett.* **135**: 121-124.
41. Porcella, A., C. Carter, D. Fage, C. Voltz, K. G. Lloyd, A. Serrano, and B. Scatton. 1991. The effect of *N*-methyl-D-aspartate and kainate lesions of the rat striatum on striatal ornithine decarboxylase activity and polyamine levels. *Brain Res.* **549**: 205-212.
42. Puttfarken, P. S., R. L. Getz, and J. T. Coyle. 1993. Kainic acid-induced lipid peroxidation: Protection with butylated hydroxytoluene and U78517F in primary cultures of cerebellar granule cells. *Brain Res.* **624**: 223-232.
43. Ransom, R. W., and N. L. Stec. 1988. Cooperative modulation of  $^3\text{H}$ -MK-801 binding to the *N*-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. *J. Neurochem.* **51**: 830-836.
44. Rao, T. S., S. J. Mick, J. A. Cler, M. R. Emmet, V. M. Dilworth, P. C. Contreras, N. M. Gray, P. L. Wood, and I. Iyengar. 1991. Effects of sigma ligands on mouse cerebellar cyclic guanosine monophosphate (cGMP) levels in vivo: Further evidence for a functional modulation of *N*-methyl-D-aspartate (NMDA) receptor complex-mediated events by sigma ligands. *Brain Res.* **562**: 43-50.
45. Resink, A., N. Hack, G. J. Boer, and R. Balàzs. 1994. Growth conditions differentially modulate the vulnerability of developing cerebellar granule cells to excitatory amino acids. *Brain Res.* **655**: 222-232.
46. Resink, A., M. Villa, D. Benke, H. Moehler, and R. Balàzs. 1995. Regulation of the expression of NMDA receptor subunits in rat cerebellar granule cells: Effect of chronic  $\text{K}^+$ -induced depolarization and NMDA exposure. *J. Neurochem.* **64**: 558-565.
47. Reynolds, I. J., and R. J. Miller. 1989. Ifenprodil is a novel type of *N*-methyl-D-aspartate receptor antagonist: Interaction with polyamines. *Mol. Pharmacol.* **36**: 758-765.
48. Sacaan, A., and K. M. Johnson. 1989. Spermine enhances binding to the glycine site associated with the *N*-methyl-D-aspartate receptor complex. *Mol. Pharmacol.* **36**: 836-839.
49. Schmutz, M., C. H. Portet, A. Jeker, K. Klebs, A. Vassout, H. Allgeier, R. Heckendorf, G. E. Fagg, H. R. Olpe, and H. Van Riezen. 1990. The competitive NMDA receptor antagonists CCP 37849 and CCP 39551 are potent, orally active anticonvulsants in rodents. *Naunyn-Schmiedebergs Arch. Pharmacol.* **342**: 61-66.
50. Schoemaker, H., J. Allen, and S. Z. Langer. 1990. Binding of  $^3\text{H}$ -ifenprodil, a novel NMDA antagonist, to a polyamine-sensitive site in the rat cerebral cortex. *Eur. J. Pharmacol.* **176**: 249-250.
51. Shalaby, I. A., B. L. Chenard, M. A. Prochniak, and T. W. Butler. 1992. Neuroprotective effects of the *N*-methyl-D-aspartate receptor antagonists ifenprodil and SL-82,0715 on hippocampal cells in culture. *J. Pharmacol. Exp. Ther.* **260**: 925-932.
52. Shaskan, E. G., and S. H. Snyder. 1973. Polyamine turnover in different regions of the rat brain. *J. Neurochem.* **20**: 1453-1460.
53. Shaw, G. G., and A. J. Paterman. 1973. The regional distribution of the polyamines spermidine and spermine in brain. *J. Neurochem.* **20**: 1225-1230.
54. Singh, L., R. Oles, and G. Woodruff. 1990. In vivo interaction of a polyamine with the NMDA receptor. *Eur. J. Pharmacol.* **180**: 391-392.
55. Slotkin, T. A., and J. Bartolomé. 1986. Role of ornithine decarboxylase and polyamines in nervous system development: A review. *Brain Res. Bull.* **17**: 307-320.
56. Sparapani, M., M. Virgili, M. Caprini, F. Facchinetto, E. Ciani, and A. Contestabile. 1996. Effects of gestational or neonatal treatment with alpha-difluoromethylornithine on ornithine decarboxylase and polyamines in developing rat brain and on adult rat neurochemistry. *Exp. Brain Res.* **108**: 433-440.

57. Tamura, Y., Y. Sato, T. Yokota, A. Akaike, M. Sasa, and S. Takaori. 1993. Ifenprodil prevents glutamate cytotoxicity via polyamine modulatory sites of *N*-methyl-D-aspartate receptors in cultured cortical neuron. *J. Pharmacol. Exp. Ther.* **265**: 1017-1025.
58. Virgili, M., M. Sparapani, F. Facchinetto, E. Ciani, R. Dall'Olio, and A. Contestabile. 1992. In vitro and in vivo evidence for spermine and spermidine neurotoxicity. *Neurodegeneration* **1**: 273-279.
59. Williams, K., C. Romano, M. A. Dichter, and P. B. Molinoff. 1991. Modulation of the NMDA receptor by polyamines. *Life Sci.* **48**: 469-498.
60. Zeevalk, G. D., and W. J. Nicklas. 1992. Developmental differences in antagonism of NMDA toxicity by the polyamine site antagonist ifenprodil. *Dev. Brain Res.* **65**: 147-155.
61. Zhang, L., X. Zheng, M. C. Paupard, A. P. Wang, L. Santchi, L. K. Friedmann, S. Zukin, and M. V. L. Bennet. 1994. Spermine potentiation of recombinant *N*-methyl-D-aspartate receptors is affected by subunit composition. *Proc. Natl. Acad. Sci. USA* **91**: 10883-10887.